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Research Article

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Structural features, molecular weight and anti-HSV activity of sulfated polysaccharides from three red seaweeds

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ABSTRACT

This study evaluated the antiviral activity of the sulfated polysaccharides (SP) from three species of seaweeds, two agarans (Acanthophora muscoides and Gracila riabirdiae) and one carrageenan (Solieria filiformis) and the relationship of this activity with the position of the sulfate groups in the structure, the sulfate content and the molecular weight. Total sulfated polysaccharides (TSP) were extracted by enzymatic digestion and fractioned on DEAE-cellulose column. The fractions with the highest yield from each species were further studied (SP-Am; SP-Gb and SP-Sf). Fourier transform infrared spectroscopy (FT-IR) demonstrated specific signs of sulfate on three species. In addition, SP-Am presented the lowest molecular weight (<100 kDa) by gel permeation chromatography. The cell viability of SP was determined by the change of the Vero cell morphology. The degree of antiviral activity was expressed as percent inhibition of the herpesvirus. The selectivity index and IC_{50} were also performed. SP-Gb (1000 µg/ml), SP-Am (250 µg/ml) and SP-Sf (500 µg/ml) showed no toxicity on Vero cells and had an antiviral effect against HSV-1 and HSV-2, when compared with the control group. The antiviral effect was mainly exerted by SP-Am, which presented the higher sulfate content and the lower molecular weight compared to SP-Gb and SP-Sf.

Key words: algae, polysaccharides, structure, molecular weight, herpes

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INTRODUCTION

Infectious diseases caused by viruses have become serious social problems worldwide in the recent years, i.e. there are increasing risks for spreading emerging and re-emerging viruses [1]. Herpes simplex viruses (HSV-1 and HSV-2) produce a wide variety of illnesses, including mucocutaneous infections, infections of the central nervous system (CNS), and an occasional infection of visceral organs; some of these conditions may be life threatening. [2]. Genital herpes is a sexually transmitted infection caused by HSV-2 and to a lesser extent HSV-1. According to the last estimation, 536 million people worldwide aged 15–49 were living with HSV-2 with an annual incidence of 23.6 million [3,4]. Immunocompetent people with genital HSV infection have frequent, painful, and recurrent genital lesions associated with intense psychosocial distress. Over the past two decades, HSV-2 infection has also been linked to three times higher risk of sexually acquired human immunodeficiency virus [5]. The first step in the infection of mucosal surfaces by viruses involves interaction between the viral glycoproteins and epitelial cell receptors of the host mucosa, because on the cell surface, heparan sulfate proteoglycans mediate the initial attachment of many viruses including herpes viruses to the target cell and the subsequent cellular entry and infection [4] Acyclovir is the most commonly used as an effective drug for HSV infection; however, it is not always tolerated

and drug resistant mutants are rapidly emerging, particularly in immunocompromised patients [6]. With the purpose of developing novel commercially available drugs, with minor adverse effects, many desirable biological activities of SP from seaweeds have been described, including antiviral effects [7-11]. The SP found in red seaweeds are mainly galactans [12]. The enantiomeric configuration of the α-galactose classifies the various galactans into two major groups, the carrageenans and the agars [13]. Previous studies determined that the chemical composition and covalent structure of galactan from the red seaweeds *A. muscoides* and *G. birdiae* are similar to an agar [14, 15] and *S. filiformis*, similar to carrageenan [16]. The biological activity of SP is related to aspects of chemical structure including degree of sulfation, molecular weight, constituent-sugar composition, conformation and dynamic stereochemistry [17]. In the case of antiviral action against HSV, several studies have shown a direct relationship between stereochemistry and the content of sulfate radicals with anti-HSV activity, but few have demonstrated results that prove the influence of molecular weight on antiviral activity. Given the above, this study aims to investigate the antiviral activity of three species of sulfated polysaccharides from red algae, two agarans (*A. muscoides* and *G. birdiae*) and one carrageenan (*S. filiformis*) and the relationship of this activity with the position of sulfate groups in the structure and also with the molecular weight.

EXPERIMENTAL SECTION

Algae

G. birdiae, *S. filiformis* and *A. muscoides* were obtained from the Atlantic coast of Brazil (Flecheiras Beach, Trairí-Ceará). After collection, specimens were taken to the Carbohydrates and Lectins Laboratory, Department of Biochemistry and Molecular Biology, Federal University of Ceará and then cleaned of epiphytes, washed with distilled water and stored at -20 °C until further use. Voucher specimens (nos. 40781, 35682 and 46093, respectively) were deposited in the Herbarium PriscoBezerra in the Department of Biology, Federal University of Ceará, Brazil.

Cells and viruses

Vero (African green monkey kidney) cells were maintained in Eagle's minimum essential medium (MEM), incubated at 37 ºC, supplemented with 3mM L-glutamine, 50 mg/mL garamicin, 2,5 mg/mL of fungizone, 0.25% sodium bicarbonate and 10% calf serum, maintained at 5% CO_2 atmosphere. Samples of HSV-1 and HSV-2 were obtained by Experimental Laboratory of Antiviral and Cytotoxic Drugs of Department of Virology/IMPG/UFRJ.

Isolation of SP

Total sulfated polysaccharides (TSP) were extracted from the red seaweeds *A.muscoides*, *S.filiformis*or *G. birdiae* by protease digestion in 0.1 M sodium acetate buffer (pH 5.0), 5 mM EDTA, 5 mM cysteine and incubated at 60 ºC for 6 h as previously described[14,16,18]. For each algal species, the TSP obtained (50 mg) was dissolved in 25 mL of 50 mMsodium acetate buffer (pH 5.0) and applied to a DEAE-cellulose column (28×2.0 cm) equilibrated with the same solution. The column was developed by a step-wise gradient of 0 → 1.5 M NaCl in the same solution with of intervals of 0.25M between each concentration. The flow rate of the column was 2.5 mL/min. Fractions of 5 mL were collected and analyzed for sulfated polysaccharide using the metachromatic assay (A_{525} nm) with 1-9 dimethyl-methylene blue (DMB) as described [19].The protocols were performed with polysaccharide fraction that showed a higher yield for each alga, called SP-Gb (*G.birdiae*), SP-Sf (*S.filiformis*) and SP-Am (*A.muscoides*).

Free sulfate content

After acid hydrolysis of the soluble polysaccharides in 1 M HCl at 110 °C for 5 h, free sulfate was measured with the gelatin–barium method previously described, using Na_2SO_4 as standard [20].

FTIR analysis

The IR spectra of the polysaccharide fractions were determined using a Fourier transform infrared spectrometer (Shimadzu IR spectrophotometer (model 8300). The polysaccharide was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets for FTIR measurement in the wavenumber range of 700 and 2000 cm^{-1} using 16 scans.

Molar weight distribution

The peak molar weight (Mpk) of the polysaccharide fractions, obtained by DEAE-cellulose ion exchange chromatography, were estimated by gel permeation chromatography with a SHIMADZU equipment using an Ultrahydrogel linear column (7,8 x 300 mm), flow 0.5 mL/min, 0.5% polysaccharide concentration and 0.1 M $NaNO_3$ as solvent. A differential refractometer and an ultraviolet photometer (at 254 nm) were used as detectors. For the calibration plot, pullulan fractions (Shodex Denko) of known molecular weight ($5,9 \times 10^3$; $1,18 \times 10^4$; $4,73 \times 10^4$; $2,12 \times 10^5$ e $7,88 \times 10^5$ g/mol) were used as standards.

Cytotoxicity assay

The cytotoxicity assay was performed by incubating Vero cell monolayers cultivated in 96 well plates with two-fold serial dilutions of the compounds in triplicate, for two days at 37 °C. The cellular viability was further evaluated by morphological changes [21] and by cellular viability using the neutral red dye-uptake method [22]. It was calculated the highest concentration of polysaccharide fraction showed no change in cell morphology called Maximum non-toxic Concentration(MNTC) and the concentration which reduces the viable cell count by 50 % (CC_{50}).

Antiviral Assay

This assay was performed in 96well plates using serial dilutions of the polysaccharide fraction (concentration based on MNTC results), followed by the addition of virus suspension(100$TCID_{50}$/ml). As positive control, cells used were grown in the presence of viral suspension and, as negative control, cells were cultured in the absence of the used virus, in the absence of the polysaccharide fraction. The degree of antiviral activity was expressed in viral inhibition index (VII) and percent inhibition (PI). The VII was obtained by the formula proposed by Mendes et al [23]: VII = B - A; where B is the titre of the virus in cell culture in the absence of the polysaccharide fraction (control) and A is the titre of the virus in cell culture in the presence of the polysaccharide fraction. The PI was calculated according to the formula proposed by Nishimura, Toku and Fukuyashu [24]: PI = [1- (T antilog/antilog C)] x 100; where T is the infectious units in the cell culture treated with the polysaccharide fraction and C corresponds to the infectious units in the untreated cell culture (control).For all viruses, the effective concentration able to inhibit 50% of viral cytopathic effect (IC_{50}) was calculated by linear regression dose response. The selectivity index (SI) was determined by the ratio CC_{50}/IC_{50}.

RESULTS AND DISCUSSION

Yield and sulfate content from SP

Enzymatic extraction of TSP from the red seaweeds *A. muscoides, G. birdiae and S. filiformis*, followed by ion exchange chromatography on DEAE cellulose column, resulted in the isolation of polysaccharidic fractions of TSP, as determined previously [14,16,18]. The fractions from each alga that provided the highest yields were used on the experiments. The chosen fractions were denominated SP-Am, SP-Gb and SP-Sf from *A. muscoides, G.birdiae* and *S.filiformis*, respectively. Initially, the yield and the sulfate content of the selected fractions were quantified (on Table 1). SP-Am presented the higher yield (54.7%) and sulfate content (26.8%) and SP-Sf presented the lower yield (6.8%) and sulfate content (12.7%).

FT-IR spectra

The FT-IR spectra of SP-Am, SP-Gb and SP-Sf are shown in Fig. 1. Typical absorption bands showed that the signals present in SP-Am and SP-Gb are corresponding to agarans and the signals present the SP-Sf are corresponding to a carrageenan. According with Melo et al. [25], the signal 1375 cm^{-1} may be attributed to the sulfate ester bond and the signals 1258 and 933 cm^{-1} correspond to vibrations S=O (ester sulfate) and C-O-C (3,6-anhydro-α-L-galactopyranose), respectively. The signal 1076 cm^{-1} is related to the structural form of a galactan. The band of 890 cm^{-1} corresponds to the sign of an agar. The bands 845, 830 and 820 cm^{-1} correspond to the presence of sulfate groups on the galactose structure in the C-4 position, C-2 and C-6, respectively and the band 805 cm^{-1} indicates the presence of sulfate group C-2 in the 3,6-anhydro-L-galactose. These signals are observed for characteristic agarocolloids, as reported by Mollet, Rahaoui and Lemoine [26]. The bands at 932, 896 and 846 cm^{-1}, indicating the presence of 3,6-anhydrogalactose, β-D-galactose-6-carbon and galactose-4-sulfate, are characteristic of carrageenan, type *kappa* [16]. In addition, the band of 770 cm^{-1} is assigned to the piranosidic ring in the backbone of both agars as for carrageenans [27]. Furthermore, the band between 1642-1406 is related with uronic acid [28] and the band 1150 cm^{-1} is attributable to vibration of C-O and C-C of piranosic ring stretching, common to all polysaccharides [29].

Molecular weight

SP-Am, SP-Gb and SP-Sf were applied to gel permeation chromatography to evaluate homogeneity and molecular weight. The equation obtained from the calibration plot was: log Mw = 14.10603 - 0.95109 Ve, where Ve is the volume of elution in ml. The linear correlation coefficient was 0.9907. The gel permeation chromatography profile demonstrated that SP-Am had a symmetrical sharp peak revealing a low molecular weight. In contrast, SP-Gb and SP-Sf presented high molecular masses. The average molecular weight of SP-Am, SP-Gb and SP-Sf were estimated to be 66, 134 and 112 kDa, respectively. In previous studies performed with polysaccharides from other species of red seaweed, it was suggested that sulfated polysaccharides, which have molecular weights above 100 kDa, are considered heterogeneous systems formed by polysaccharide chains with high molecular weights [30-32]. Therefore, SP-Gb and SP-Sf have high molecular weight and SP-Am presented a low molecular weight.

Citotoxicity and Cellular Viability

Cytotoxicity assay is the first test to be performed to evaluate the biocompatibility of new compounds for use as biomedical tools. After confirmation of the low toxicity of the compound, biocompatibility studies *in vitro* and *in vivo* may proceed. The most common parameter used to evaluate *in vitro* toxicity is cell viability, which can be evidenced with the use of vital dyes such as neutral red, that distinguishes between living, dead or damaged cells by color intensity measurement of the cell culture by light microscopy [33]. Herein, Vero cells (5 x 10^5 cells/well) were submitted to different serial dilutions of SP-Am, SP-Gb and SP-Sf (1000 µg ml - 3.9 mg/ml). The results showed that in all concentrations, except 1000 µg/mL, cells were viable. As the assay was performed in concentration from 1000 µg/mL, we suggest that the CC_{50} obtained for these cell lines is considered greater than 1000, 500 and 250 µg/ml from SP-Gb, SP-Sf and SP-Am, respectively. The MNTC obtained for the SP-Gb and SP-Sf was 500 and 62.5 µg/ml from SP-Am, considering that the cell viability obtained was greater than 80% (Table 2).

Anti-HSV activity

Based on previous studies of antiviral effect of SP from seaweed against enveloped viruses such as herpes simplex [34], we evaluated the antiviral effect of SP-Gb, SP-Am and SP-Sf against HSV-1 and HSV-2. SP fractions on their MNTC, showed a significant inhibition of the cytopathic effect against both serotypes. Based on this result, data of inhibition of cytopathic effect of SP fractions were calculated taking into account only the lowest concentration. Therefore, in this concentration, SP-Gb, SP-Am and SP-Sf presented for HSV-1 a VII of 0.75, 1.63 and 0.6, generating PI of 82.2, 97.6 and 74.6%, respectively. For HSV-2, SP-Gb, SP-Am and SP-Sf presented a VII of 1.25, 3.5 and 1.6, generating a PI from 94.4, 99.9 and 97.5%, respectively (Table 3). These data are in agreement with the literature that relates the fundamental mechanism for anti-HSV activity of SP of algae with the inhibition of virus adsorption, the initial step in viral replication process [17,35]. This type of viral inhibition is justified because an infection caused by HSV promotes interaction between the viral glycoprotein C (gC) and heparan sulfate molecules on cell surface, although glycoprotein B may contribute to this function [4]. Thus, the algal SP may act competitively to the binding site gC, preventing the viral adsorption process [8].Bandyopadhyay et al. [10] suggested that the antiviral effect may be related to the presence of sulfate groups that interact directly with the viral particle. As the selectivity index (SI) of a test compound represents the degree of safety for its use, this parameter was determined for the fractions, using the ratio between CC_{50} and IC_{50}. The results showed IC_{50} values for SP-Gb, SP-Am and SP-Sf of 98.8, 3.6 and 15.6 for HSV-1; and 20.5, 9.0 and 14.8 for HSV-2, respectively. In relation to SI, the ratio showed that, for HSV-1, SP-Am presented the major value, however, this is not observed for HSV-2, because SP-Gb presented the highest value of CC_{50}, representing a better SI when compared to SP-Am and SP-Sf even with a lower value of IC_{50} (Table 4).

Structure/activity relationships

The carbohydrate backbone of red algal SP is usually composed of alternating 4-linked α-galactose and 3-linked β-galactose. The structural heterogeneity of these polysaccharides mostly arises from variable sulfate ester and/or methyl ether substitutions, occasional occurrence of 3,6-anhydro-α-galactosyl units [36].Fraction SP-Am, with a molecular weight of 66 kDa, presented a heterogeneous structure identified on FT-IR with sulfate esters (1260 cm^{-1}) and partial conversion of α-galactose units into 3,6-anhydro-α-galactose (935 cm^{-1}) and galactose 6-sulfate (820 cm^{-1}), featuring a compound with significant sulfate content (26.8%), confirmed through free sulfate percentage (Table 1). In contrast, the fraction SP-Gb had a higher molecular weight (123 kDa) and the sulfate content was also different. However, sulfate groups, if present, were located mostly at the same place with SP-Am, but with a lower intensity. Therefore, these chemical features justify the higher antiviral potency of SP-Am in comparison to SP-Gb. With respect to the SP-Sf, this fraction showed moderate activity against HSV-2, with IC_{50} values between 14.8 and 15.6 µg/mL. Perhaps the relatively similar average molecular mass of SP-Sf (112 kDa) compared to SP-Gb (123 kDa) and their similar sulfate contents (12.7 and 14.8%, respectively) and sugar compositions (galactose as the major sugar) are responsible for exhibiting similar potency toward HSV-2. Given the lack of cytotoxicity of these polymers, the values of SI, defined as the CC_{50}/IC_{50} ratio, obtained for all tested fractions were considered good, when compared to other studies involving SP from red algae [1, 37]. Particularly, the products derived from SP-Am presented SI higher than 69.4 against HSV-1 and could be considered very effective and selective inhibitors compared to SP-Gb and SP-Sf. Therefore, the anti-HSV-1 activities of the SP of present study are related to high charge density and the molecular weight.

Table 1. Yield and sulfate content of sulfated polysaccharides from the red seaweeds

Fractions	Yield (%)	Free sulfate (%)
SP - Gb	23.6	14.8
SP - Am	54.7	26.8
SP - Sf	6.8	12.7

Table 2: Cell viability of three sulfated polysaccharides on Vero cells

Fractions	MNTC (µg/mL)	CC_{50} (µg/mL)
SP - Gb	500	>1000
SP - Am	62.5	>250
SP - Sf	500	>500

**MNTC= Maximum non-toxic concentration; CC_{50} = 50% cytotoxic concentration to the cultured cells*

Table 3: Viral inhibition index and percent of inhibition of sulfated polysaccharides against HSV-1 and HSV-2 on Vero cells

Fractions	HSV-1		HSV-2	
	VII	PI (%)	VII	PI (%)
SP - Gb	0.75	82.2	1.25	94.40
SP - Am	1.63	97.6	3.5	99.99
SP - Sf	0.6	74.9	1.6	97.50

**HSV-1 = Herpes simplex virus type 1; HSV-2 =Herpes simplex type 2virus;VII =Viral Inhibition index(Difference virus titer in the presence and absence of the substance); PI=Percent of Inhibition.*

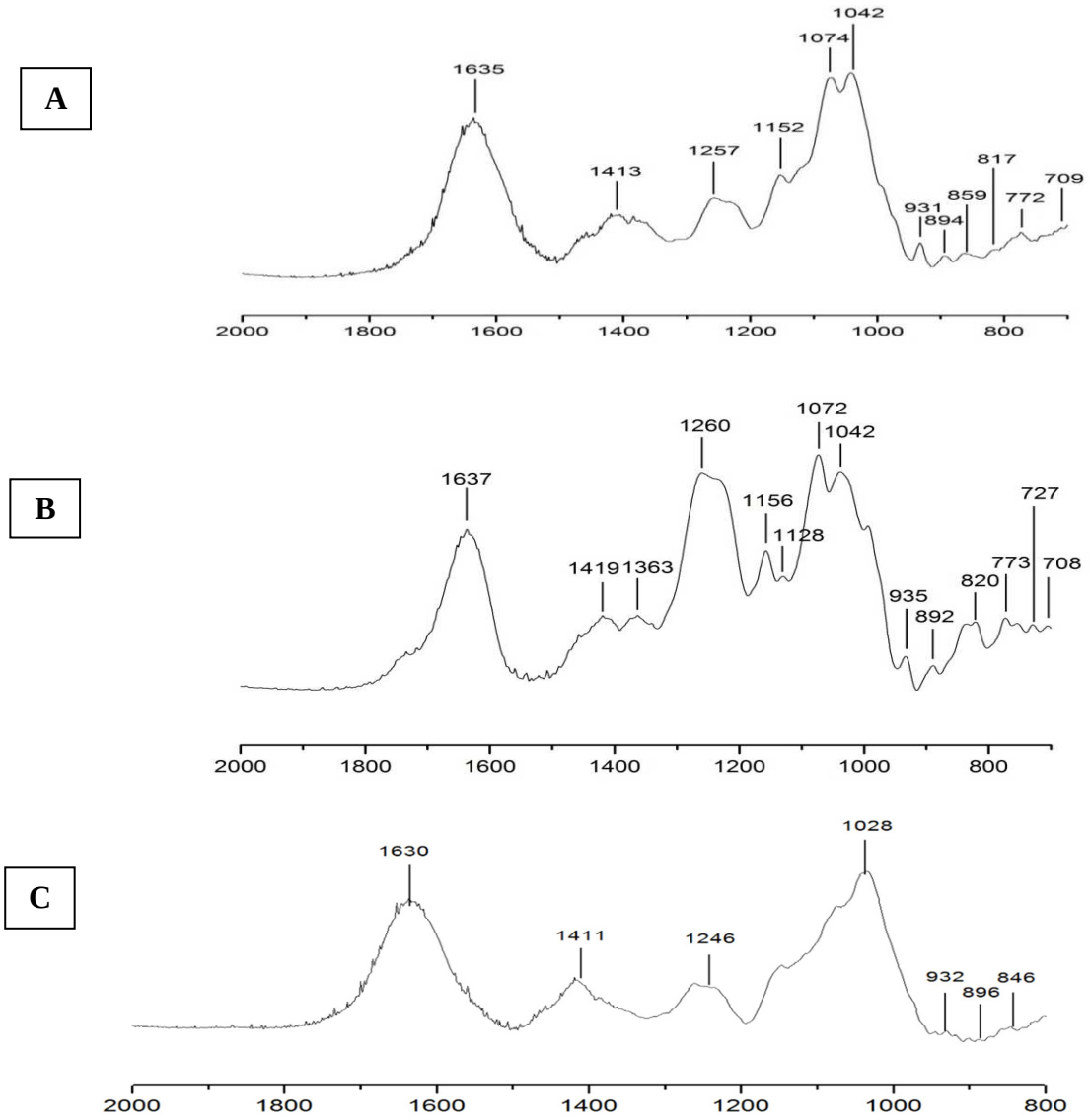

Figure 1 - Infra red spectra (700–2000 cm^{-1}) of sulfated polysaccharides from *Gracilariabirdiae* (A), *Acanthophoramuscoides* (B) and *Solieriafiliformis*. (C)

Table 4: Antiviral activity of sulfated polysaccharides against HSV-1 and HSV-2 on Vero cells

Fractions	CC_{50}	HSV-1	HSV-2	HSV-1	HSV-2
		IC_{50} (µg/mL)	IC_{50} (µg/mL)	SI	SI
SP - Gb	> 1000	0.75	82.2	1.25	94.40
SP - Am	> 250	1.63	97.6	3.5	99.99
SP - Sf	> 500	0.6	74.9	1.6	97.50

**CC_{50} = Cytotoxic concentration 50% ; IC_{50} = Inhibitory concentration 50% and SI = Selectivity index*

CONCLUSION

The results reported here demonstrate the antiviral effects of SP isolated from three red seaweeds species. The antiviral potency of the SP was presented independently of the enantiomeric sugar composition. The effect was mainly exerted by the sulfate content and molecular weight, because the SP fraction (SP-Am), with lower molecular weight and higher sulfate content, presented the better effect against herpes virus (HSV-1). Furthermore, this work warrants related studies on SP from other seaweeds aiming to increase the knowledge of novel antiviral drugs, their mechanism of action and critical structural features required for this activity.

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